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Laboratory Studies of Disinfectants Against *Legionella pneumophila*†

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Legionella pneumophila suspended in tap water was exposed to biocides recommended for inhibiting biological growth in cooling towers and evaporative condensers of air-conditioning systems. Chlorine, 2,2-dibromo-3-nitrilopropionamide, and a compound containing didecyldimethylammonium chloride and isopropanol were effective in destroying concentrations of 10^5 to 10^6 viable cells per ml. Formulations consisting of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one, disodium ethylene bis(thiocarbamate) and sodium dimethyl dithiocarbamate, and a phenolic with pentachlorophenate and sodium salts of other chlorophenols were less effective.

In the course of epidemiological investigations of Legionnaires disease outbreaks, a bacterial species, *Legionella pneumophila*, responsible for the disease, has been recovered from water in cooling towers and evaporative condensers of buildings associated with cases (2, 5). Epidemiological data suggest that infection may have been acquired as a result of inhaling infectious aerosols of contaminated water generated from these sources. Prompt treatment of the contaminated water with effective bactericidal agents is of special concern in the control of such outbreaks.

At present, various methods and chemical microbicides are being used to prevent accumulation of biological growth in water of comfort-type air-conditioning systems. Studies have shown that *L. pneumophila* is susceptible to several types of antiseptics and disinfectants used primarily in hospitals for tissue and surface decontamination (7). The toxicity of formulations specifically recommended for inhibiting growth of algae, bacteria, and fungi in cooling-tower and evaporative-condenser water has not been demonstrated for *L. pneumophila*. An investigation was undertaken to determine whether several commerical disinfectants currently registered by the U.S. Environmental Protection Agency and used for inhibiting biological growth in water of cooling towers and evaporative condensers could be used in an outbreak situation to destroy waterborne *L. pneumophila*. The disinfectants were tested under uniform laboratory conditions solely to determine their selective toxicity for *L. pneumophila* in tap water.

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MATERIALS AND METHODS

Test organisms. All tests were performed with the Philadelphia 1 strain of *L. pneumophila* which was originally recovered from lung tissue of a patient (6). Since repeated subculturing on artificial media may affect the resistance of organisms to germicidal action, the strain was maintained in guinea pig spleen tissue that had been macerated and stored at −70°C. For each test, frozen spleen tissue was quick-thawed and cultured on charcoal-yeast extract (CYE) agar (3) at 37°C in an atmosphere of 2.5% CO_2. After 6 to 7 days of incubation, growth was harvested, washed twice in sterile, Cl_2-free tap water, and suspended in sterile tap water by vigorous shaking to break up aggregates. The suspension of washed cells was adjusted turbidimetrically to a concentration of 10^7 to 10^8 cells per ml.

Disinfectants. Disinfectants selected for this study were commercially prepared and recommended for use in controlling biological growth in cooling towers and evaporative condensers of comfort-type air-conditioning systems. The types and percentages of active ingredients and total product concentration of each disinfectant tested are shown in Table 1. Inert ingredients that may have been added in preparation of the final compound were not listed in the descriptive literature. The total product concentrations tested were the initial concentration (slug dose) and maintenance concentration recommended by the manufacturer and 50% less than the maintenance concentration. Susceptibility to calcium hypochlorite was tested in terms of free chlorine rather than as total product concentration. Pretest free chlorine was measured by the amperometric method, after test by the *N,N*-diethyl-*p*-phenylenediamine method (1). The desired concentrations of disinfectants and neutralizers were prepared with chlorine-free, chlorine-demand-free tap water immediately before the addition of washed cells.

Test procedures. The bactericidal action of each concentration was measured by exposing 10^5 to 10^7 viable cells per ml in 500 ml of disinfectant solution contained in a 1-liter Erlenmeyer flask. Immediately after cells were added, the suspension was thoroughly agitated, and 9.0 ml, designated as sample taken at

TABLE 1. *Type and concentration of disinfectant tested*

Disinfectant	Concn (mg/1,000 ml)	Active ingredients, inert ingredients, and neutralizer	%
A	8.9, 17.8, 71.0	Sodium pentachlorophenate	9.5
		Sodium salts of other chlorophenols	1.2
		Inert ingredients	89.3
		Neutralizer: soy lecithin	0.07
		Tween 60	0.5
B	6.0, 12.0, 24.0	2,2-Dibromo-3-nitrilopropionamide	20
		Inert ingredients	80
		Neutralizer: sodium thiosulfate	0.1
C	20.0, 40.0, 120.0	Disodium ethylene bis(thiocarbamate)	15
		Sodium dimethyl dithiocarbamate	15
		Inert ingredients	70
		Neutralizer: skim milk	10
D	3.3, 6.6[a]	Calcium hypochlorite	65
		Inert ingredients	35
		Neutralizer: sodium thiosulfate	0.1
E	72.0, 144.0, 648.0	Didecyldimethylammonium chloride	50
		Isopropanol	20
		Inert ingredients	30
		Neutralizer: soy lecithin	0.07
		Tween 60	0.5
F	20.0, 60.0, 120.0	5-Chloro-2-methyl-4-isothiazolin-3-one	8.6
		2-Methyl-4-isothiazolin-3-one	2.6
		Inert ingredients	88.8
		Neutralizer: sodium thioglycolate	0.1

[a] Free chlorine.

zero time, was transferred to 1.0 ml of the appropriate neutralizer (Table 1). In preliminary experiments the neutralizers exhibited no toxicity for *L. pneumophila*. A 1.0-ml aliquot of the neutralized sample was serially diluted (1:10) with sterile tap water. From each of four suitable dilutions, 0.1 ml was cultured in duplicate on CYE agar at 37°C in an atmosphere of 2.5% CO_2.

Test suspensions were then incubated at 25°C. The number of survivors was subsequently determined after 3, 6, 24, and 168 h. At each time interval, a 9.0-ml sample was processed as described above.

The controls consisted of 10^5 to 10^7 cells per ml in 500 ml of sterile tap water without disinfectant that were exposed to the same conditions as the test suspensions.

The number of survivors was determined in terms of the number of *L. pneumophila* colonies developing on CYE agar after 6 to 7 days of incubation at 37°C in an atmosphere of 2.5% CO_2. Plates on which colonies did not develop were incubated for 14 days before being discarded.

An attempt to quantitatively confirm the results obtained with the direct plating method by washing exposed cells on membrane filters and culturing on Feeley-Gorman agar (4) was unsuccessful due to the failure of *L. pneumophila* to grow consistently on the filters.

The validity of data obtained with the dilution plate count procedure was confirmed with an in vivo method, using embryonated chicken eggs. Preliminary experiments demonstrated that the disinfectants, neutralizers, and neutralizer-disinfectant combination did not exhibit tissue toxicity at the concentrations used in this study. Undiluted control and test suspensions, with and without neutralizer, were inoculated into the yolk sac of 7-day-old chicken embryos. For each determination 0.25 ml was inoculated into each of six eggs. The yolk sacs of chicken embryos that died 3 to 14 days after inoculation were cultured on CYE agar to confirm that death was caused by proliferation of *L. pneumophila*.

RESULTS

Resistance of *L. pneumophila* to the lethal action of disinfectants tested is shown in Tables 2 and 3. The most effective bactericidal action was observed with concentrations of calcium hypochlorite that yielded 3.3 and 6.6 μg of free chlorine per ml. A total of 1.3×10^6 viable cells per ml were rapidly inactivated. No survivors were recovered, either on artificial media or in chicken embryos, from samples cultured immediately after the washed cells were added to the disinfectant solution or after exposures of 3, 6, and 24 h. No tests were performed at 168 h.

The combination of didecyldimethylammonium chloride and isopropanol was also highly effective. Of the 2.0×10^6 viable cells per ml exposed, only a few survivors were recovered at zero time. Single colonies developed from an undiluted sample of cells exposed to 72.0 μg/ml and a 10^{-1} dilution of cells exposed to 144.0 μg/ml. These samples also infected chicken embryos. After exposures of 3, 6, 24, and 168 h, no viable cells were recovered.

The lethal action of 2,2-dibromo-3-nitrilopropionamide was not as rapid as that observed for

TABLE 2. *Log decrease of L. pneumophila exposed to disinfectants*

Disinfectant[a]	Concn (mg/1,000 ml)	Log of no. of survivors at:				
		0 h	3 h	6 h	24 h	168 h
A	Control	5.7	5.9	6.0	5.9	5.8
	8.9	5.8	5.8	5.6	5.7	5.0
	17.8	5.7	5.8	5.8	5.3	3.3
	71.0	5.6	5.6	5.5	4.4	0.0
B	Control	6.5	6.4	6.5	6.4	6.2
	6.0	6.1	3.0	1.5	0.0	0.0
	12.0	5.9	1.2	0.7	0.0	0.0
	24.0	5.5	0.0	0.0	0.0	0.0
C	Control	5.1	5.1	5.2	5.3	5.2
	20.0	5.3	5.2	5.2	4.9	1.7
	40.0	5.2	5.2	5.2	4.9	0.0
	120.0	5.2	5.1	5.3	3.3	0.0
D	Control	6.1	6.0	6.2	5.9	NT[b]
	3.3	0.0	0.0	0.0	0.0	NT
	6.6	0.0	0.0	0.0	0.0	NT
E	Control	6.3	6.3	6.4	6.4	6.3
	72.0	1.0	0.0	0.0	0.0	0.0
	144.0	1.0	0.0	0.0	0.0	0.0
	648.0	0.0	0.0	0.0	0.0	0.0
F	Control	6.4	6.4	6.4	6.4	6.2
	20.0	6.3	5.3	4.8	1.4	0.0
	60.0	6.3	4.2	3.9	0.0	0.0
	120.0	6.4	4.2	2.9	0.0	0.0

[a] A, Sodium pentachlorophenate; B, dibromonitrilopropionamide; C, thiocarbamate; D, chlorine; E, quaternary ammonium; F, isothiazolin.
[b] NT, Not tested.

the calcium hypochlorite or the quaternary-alcohol combination. At zero time there was very little inactivation of the 3.5×10^6 viable cells per ml exposed. After exposures of 3 and 6 h relatively few cells survived in the 6.0- and 12.0-μg/ml concentrations, and none were recovered from the 24.0-μg/ml test suspension. Infection of chicken embryos was consistent with results observed on CYE agar.

A total of 2.3×10^6 viable cells per ml were exposed to 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one. After 6 h, approximately 98.0% of the population was destroyed by 20 μg and more than 99.0% was destroyed by 60.0 and 120.0 μg of disinfectant per ml. After 24 h, viability on artificial medium was demonstrated only for cells exposed to the lowest concentration, 20 μg/ml; however, chicken embryos were infected with inocula from all concentrations. No viable cells were recovered after 168 h of exposure on artificial medium or from chicken embryos.

Viable cells were recovered up to 24 h on CYE agar and 168 h in chicken embryos from suspensions of 20, 40, and 100 μg of disinfectant per ml containing disodium ethylene bis(dithiocarbamate) and sodium dimethyl dithiocarbamate. On CYE agar viability at 168 h was demonstrated only for cells from the 20.0-μg/ml concentration. The initial count of viable cells exposed was 1.8×10^5 cells per ml.

The disinfectant containing pentachlorophenate and sodium salts of other chlorophenols inactivated approximately 39, 73, and 97% of 5.3×10^5 cells exposed to 8.9, 17.8, and 71.0 μg/ml, respectively, after 24 h of exposure. At 168 h, only the 71.0-μg/ml concentration did not yield viable cells on artificial medium and in chicken embryos.

DISCUSSION

In an epidemic situation of Legionnaires disease, the capacity of contaminated environmental sources to cause infection must be eradicated without delay. Water in cooling towers and evaporative condensers suspected as a source of infection should be promptly treated with disinfectants possessing rapid and effective bacteri-

TABLE 3. *Recovery of L. pneumophila from yolk sac of chicken embryos inoculated with suspensions of L. pneumophila exposed to disinfectants*

Disinfectant[a]	Concn (mg/1,000 ml)	Chicken embryos positive for *L. pneumophila*				
		0 h	3 h	6 h	24 h	168 h
A	Control	+	ND[b]	ND	+	+
	8.9	+	+	+	+	+
	17.8	+	+	+	+	+
	71.0	+	+	+	+	−
B	Control	+	ND	ND	ND	+
	6.0	+	+	+	−	−
	12.0	+	+	−	−	−
	24.0	+	−	−	−	−
C	Control	ND	+	ND	ND	+
	20.0	+	+	+	+	+
	40.0	+	+	+	+	+
	120.0	+	+	+	+	+
D	Control	+	+	+	+	ND
	3.3	−	−	−	−	ND
	6.6	−	−	−	−	ND
E	Control	+	+	+	+	+
	72.0	+	−	−	−	−
	144.0	+	−	−	−	−
	648.0	−	−	−	−	−
F	Control	+	+	+	+	+
	20.0	+	+	+	+	−
	60.0	+	+	+	+	−
	120.0	+	+	+	+	−

[a] A, Sodium pentachlorophenate; B, dibromonitrilopropionamide; C, thiocarbamate; D, chlorine; E, quaternary ammonium; F, isothiazolin.
[b] ND, Not determined.

cidal action. Of the disinfectants tested, formulations containing calcium hypochlorite or didecyldimethylammonium chloride and isopropanol as active ingredients demonstrated satisfactory bactericidal action against *L. pneumophila* in water. Although 2,2-dibromo-3-nitrilopropionamide did not act as rapidly as free chlorine or the quaternary-alcohol combination, failure to recover viable cells after 6 h of exposure suggests that this compound would also destroy the organism in water within a reasonable treatment period.

The disinfectant containing 5-chloro-2-methyl-4-isothiazolin-3-one with 2-methyl-4-isothiazolin-3-one exhibited slower bactericidal action than the quaternary, hypochlorite, or 2,2-dibromo-3-nitrilopropionamide compounds. Effective action against *L. pneumophila* in cooling-tower water would require exposure periods of up to 24 h. On the basis of results obtained under conditions of this laboratory study, germicides containing disodium ethylene bis(dithiocarbamate) with sodium dimethyldithiocarbamate or the sodium pentachlorophenate and sodium salts of other chlorophenols appear to be of limited value in outbreaks associated with contaminated water in air-conditioning systems. Viable cells of *L. pneumophila* were recovered even after 24-h exposure periods.

The results obtained by methods used in this study indicate relative toxicities of commerically available disinfectants currently recommended for controlling fungi, bacteria, and algae in water of cooling towers and evaporative condensers. Formulations containing calcium hypochlorite, didecyldimethylammonium chloride, and alcohol or 2,2-dibromo-3-nitrilopropionamide satisfactorily inhibited *L. pneumophila*. Under practical conditions, bactericidal effectiveness cannot be based solely on results obtained in the laboratory. Diverse physical, chemical, and biological conditions that may exist in operating air-conditioning systems can markedly affect bactericidal action. For example, *L. pneumophila* was highly susceptible to free chlorine released from calcium hypochlorite. However, chlorine is especially unstable in the presence of organic matter and may be rapidly neutralized. To obtain levels of free chlorine necessary to inhibit *L. pneumophila* under actual conditions, excessive concentrations of calcium hypochlorite may be required. High concentrations of the disinfectant could result in prohibitive corrosive action. Although there was no attempt in this study to vary temperature, organic matter, extraneous chemical substances, proportions of test organisms to disinfectant, or other conditions that influence bactericidal efficiency, such factors must be considered when selecting a chemical disinfectant for treating contaminated water in air-conditioning systems suspected as a source of *L. pneumophila*. Studies to determine the effectiveness of some of these biocides in cooling towers contaminated with *L. pneumophila* are planned.

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